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Dynamic Alterations of Replication Timing in Mammalian Cells

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Summary

Background: The eukaryotic genome is divided into distinct replication timing domains, which are activated during S phase in a strictly conserved order. Cellular differentiation can alter replication timing in some loci, but recent experiments yielded conflicting data regarding the relationship between gene expression and replication timing. The genetic and epigenetic determinants of replication timing in mammalian cells have yet to be elucidated.
Results: We developed a mammalian experimental system in which the timing of DNA replication can be altered in a controlled manner. This system utilizes sequences from the human β-globin locus that exhibit orientation-dependent transcriptional silencing when inserted into the murine genome. We found that before insertion, the murine target site replicated late during S phase. After insertion, replication timing depended on the orientation of the transgene. In a transcription-permissive orientation, the transgene and flanking sequences replicated early. In the reverse (silencing-prone) orientation, these sequences replicated late. Early replication correlated with histone modifications of the transgene chromatin but could be observed in the absence of the β-globin promoter. Importantly, the replication timing switch did not require a replication origin within the transgene.
Conclusions: Transgene insertions into mammalian heterochromatin can alter the timing of DNA replication at the insertion site. This differentiation-independent replication timing switch did not necessitate insertion of an active promoter or a replication origin. These observations suggest that the timing of DNA replication can be manipulated by changes in DNA sequence, but that the determinants of replication timing are distinct from the sequences that specify replication initiation sites.

Introduction

Mammalian cells initiate DNA replication through a series of coordinated steps that ensure accurate replication of the entire genome during each cell cycle. Previous studies have demonstrated that the mammalian genome is divided into distinct chromosomal replication timing domains [1]. The replication timing plan involves sequential activation of genomic domains, which occurs in a strictly conserved order. In mammalian cells, long-range mapping of replication timing suggests that replication timing zones are extensive chromosomal regions and that the transition between early and late replication occurs gradually, over a region of several megabases [2]. Earlier-replicating domains in mammalian cells tend to be gene-rich regions, whereas heterochromatin replicates later [1, 3–5]. Whole genome analyses of transcription and replication timing suggest that early replication does not correlate with transcription in the yeast *Saccharomyces cerevisae* [6] and *Schizosaccharomyces pombe* [7]. In some cases, late replication in yeast correlates with the proximity to chromosomal regions that undergo transcriptional silencing, such as telomeres and mating type loci [8, 9], but some heterochromatic regions in the yeast *S. pombe* replicate early [7]. Interestingly, a global correlation between replication timing and gene expression was recently observed in *Drosophila* [4]; this finding supports the hypothesis that gene expression and replication timing are correlated in metazoa. These data imply that the correlation between replication timing and gene expression may be a feature of higher eukaryotes. Aberrations in replication timing are associated with a loss of imprinting [10, 11], activation of cell cycle checkpoints [12], and genomic instability [13, 14].

While replication timing is constant within a particular tissue, some loci may exhibit tissue-specific replication timing. Activation of silent genes correlates with a switch to early replication, as shown in the human β-globin [5, 15], immunoglobulin [16], CD8 [17], and T cell receptor [18] loci. Similarly, the inactivation of X chromosomes delays the replication of the inactive chromosome during embryogenesis [19]. These observations suggest that replication timing can be regulated dynamically and can be modulated in a tissue-specific manner. Nevertheless, the precise determinants of replication timing, particularly the nature of the sequences that are responsive to putative replication timing modulators, have not been elucidated.

The human β-globin locus is a particularly attractive model for studying the *cis*-acting determinants of replication timing and the correlation between gene expression and DNA replication. This locus replicates early during S phase in preerythroid cells that express globin. In nonerythroid cells that do not express globin, the locus replicates late [3, 5]. In the human locus, both early and late replication initiate from a region (initiation region, IR) that lies between the two adult β-like globin genes [20]. A 40-kb region upstream of the globin gene cluster that includes a series of DNase I hypersensitive sites known as the locus control region (LCR) is required for initiation from the IR [21]. LCR is also required for enhanced expression of the globin genes and for early replication of the globin locus in erythroid cells [22]. However, the specific role of the LCR in establishing replication initiation and replication timing is unclear. The IR can initiate early replication at simian ectopic

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sites in the presence or absence of the LCR [23]; this finding suggests that other sequences may substitute for the function provided by the LCR in the native locus. It has been shown that LCR sequences, but not a collection of the sequences derived solely from the hypersensitive site "core" sequences, can dictate tissue-specific replication timing at ectopic positions in transgenic mice [24], but studies at the native sites suggest that the hypersensitive sites are neither essential for tissue-specific expression of the globin genes in the native locus [25] nor for early replication [26]. Nevertheless, it is important to note that, to date, all studies of LCR mutants and ectopic insertions demonstrated a correlation between early replication and a decondensed chromatin conformation [21, 26].

To investigate the relationship between DNA replication and gene expression and the roles played by the IR and LCR in these processes, we developed an experimental system in which the replication timing and gene expression can be altered in a controlled manner. This system uses sequences from the human β-globin locus inserted at a site in the murine genome through recombinase-mediated cassette exchange (RMCE) [27]. This transgene exhibits orientation-dependent silencing at some insertion sites: it is silenced when inserted in one orientation, but not in the other [28]. Orientation-specific silencing is associated with hypermethylation of the transgene DNA, but not with changes in DNase I hypersensitivity of the LCR-derived sequences inserted in the transgene. Here, we show that the insertion site, which was susceptible to orientation-dependent silencing, exhibited late replication during the S phase of the cell cycle. The insertion of an active gene facilitated early replication of the transgene and an extended chromosomal region straddling the insertion site in a reversible, orientation-dependent manner. Our findings further suggest that establishment of replication timing domains involves epigenetic changes in chromatin structure and that the determinants of replication timing are distinct from the determinants of replication initiation sites.

Results

A Late-Replicating Insertion Site for Transgenes in Murine Erythroleukemia Cells

We analyzed the replication timing of a transgene containing hypersensitive sites from the human β-globin locus that is fused upstream of the β-globin promoter and drives enhanced green fluorescent protein (EGFP) expression [28]. The hypersensitive sites included in this construct were derived from the core sequences from the LCR hypersensitive sites 2, 3, and 4 (for construct information, see [28]). The transgene exhibited orientation-dependent expression when inserted into a genomic site termed the random locus 4 (RL4) in MEL cells [28]. Insertion of the expression cassette in one orientation (permissive) exhibited stable EGFP expression, whereas when it was inserted in the other orientation (silenced), the transgene became silenced within a few weeks after transfection (Figure 1A). Loss of gene expression was accompanied by changes in DNA methylation but had no effect on the DNase sensitivity of chromatin around the LCR [28].

We assessed the replication timing of sequences adjacent to the RL4 site in MEL cells, prior to and following insertion of the transgene cassettes. Figure 1B shows a schematic representation of a replication timing analysis. We used the thymidine analog bromodeoxyuridine (BrdU) to label replicating DNA in asynchronous proliferating MEL cells, and then fractionated the labeled population according to DNA content. We then used real-time quantitative PCR with primers spanning the insertion region (see Table S1 in the Supplemental Data available with this article online) to measure the abundance of specific genomic sequences in BrdU-substituted DNA from the six isolated fractions (one G1 fraction, four fractions from consecutive stages of S phase designated S1–S4, and one G2/M fraction). (Examples of the real-time PCR output and a representative calibration curve, as well as an example of the data processing, are shown in Figures S1A and S1B and Table S2).

We first determined the replication timing of DNA sequences that lie adjacent to the RL4 site in MEL cells that had not been transfected (Figure 1A, I). The data from this analysis are summarized in Figure 2A. Sequences from early-replicating loci, such as the murine β-globin locus, were enriched in early S phase BrdU-substituted DNA (including the G1 fraction, which could contain some cells in early S phase). In contrast, sequences known to replicate late in MEL cells, such as the murine amylase locus, which is not expressed in MEL cells, were enriched in the late S phase fractions. Sequences from the insertion site were preferentially amplified in the late S phase fractions, exhibiting a pattern similar to that observed for the late-replicating amylase. These data suggested that this region replicated late during the S phase of the cell cycle.

Insertion of a Transgene Altered Replication Timing from Late to Early

We next determined the replication timing of sequences from the vicinity of the insertion site after insertion of a transgene that contained the hygromicin antibiotic resistance and the thymidine kinase gene flanked by two inverted copies of a LoxP recognition site (Figure 1A, II; see [28]). As shown in Figure 2B, cells that contained the transgene replicated the insertion site and sequences in adjacent regions throughout S phase, with the highest abundance of newly replicated DNA occurring in both the early and late S phase. This altered replication pattern was exhibited by primer/probe combinations from an extended genomic region straddling the insertion site. The murine β-globin and the murine amylase markers continued to replicate early and late during S phase, respectively. These findings suggested that after insertion there was a distinct difference between the two chromosomal copies of the RL4 site: the chromosomal region that contained a transgene replicated early, while the allelic region that lacked a transgene continued to replicate late during the S phase of the cell cycle.

A Transgene Containing Sequences from the LCR and the Globin Promoter Replicated in an Orientation-Dependent Manner

We next determined the replication timing of the transgene expressing the LCR-globin promoter-EGFP cas-

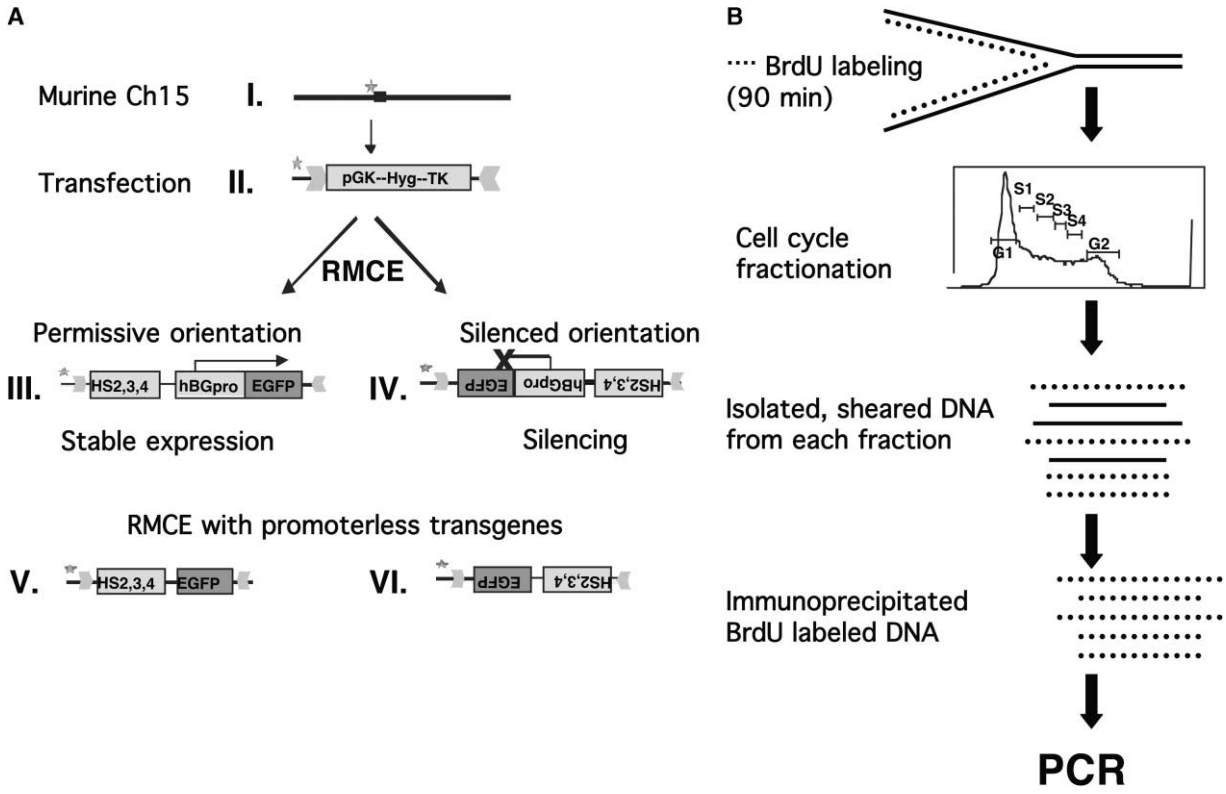

Figure 1. A Schematic Illustration of the Experimental System Used in This Study

(A) Transgene constructs whose replication timing profiles are reported here are identified by roman numerals from I to VI. The initial transfection (from I to II) inserted a transgene cassette expressing the hygromicin resistance (hyg) and thymidine kinase (TK) genes, driven by the pGK promoter (pGK), between two inverted LoxP sites into the insertion site in MEL cells. The insertion site is illustrated as a square in the upper diagram. The star represents a probe lying 2 kb 5′ to the insertion site (INsite probe). After establishment of the transgenic cell line, recombinase-mediated cassette exchange (RMCE) was used to replace this transgene with a cassette consisting of hypersensitive sites (HS) 2, 3, and 4 from the human β-globin LCR (HS2,3,4) fused to the human β-globin promoter (hBGpro) and driving EGFP expression. One of the orientations of the transgene (III) is permissive for constitutive expression, whereas the other (IV) is subject to silencing. The position of the INsite probe (star) is indicated for orientation. The lower pair of transgenes, V and VI, contained the LCR hypersensitive sites, but not the human β-globin promoter, and were inserted in the permissive and silent orientation, respectively.

(B) A schematic representation of the replication timing assay. The abundance of specific sequences in newly replicated DNA was determined by performing PCR on sheared, newly replicated DNA derived from cells isolated from various stages of the cell cycle after BrdU treatment, as described in the Experimental Procedures.

settes that replaced the antibiotic resistance marker [28]. These analyses were performed in cell lines harboring the LCR-globin promoter-EGFP cassette in both the permissive and the silent orientation (Figure 1A, III and IV). As shown in Figure 3, primers from the EGFP gene (Figure 3A) and the human globin promoter (Figure 3B) preferentially amplified sequences from BrdU-substituted DNA from early S phase when inserted in the permissive orientation; this finding suggests that the transgene replicated early during S phase. However, in the silent orientation, these primers were preferentially amplified in the late S phase fractions. The replication timing profiles of the early-replicating murine β-globin locus (Figure 3C) and the late-replicating murine amylase locus (Figure 3D) were similar in cells harboring transgenes in both orientations.

Our data further showed that cells that contained the transgene in the silenced orientation replicated the insertion site, and chromosomal sequences surrounding it, during late S phase. Therefore, the cells reverted to preinsertion replication timing (Figures 3E and 3F). Cells that contained the transgene in the permissive orientation replicated the insertion site throughout S phase, again indicating a difference between the two chromosomal alleles of these sequences. These observations were confirmed with other primers flanking the insertion site (see Figure S2 in the Supplemental Data available with this article online). These data suggested that the early replication pattern was conserved after recombinase-mediated cassette exchange if the transgene was inserted in the permissive orientation, but silencing was accompanied by reversion to late replication of both the transgene and the flanking sequences.

A Transgene Containing a Deletion of the β-Globin Promoter Exhibited Orientation-Dependent Replication Timing Patterns

We next determined the replication timing of the transgene harboring an LCR-EGFP cassette that lacked the β-globin promoter, inserted in both the permissive and the silent orientations (Figure 1A, V and VI). As shown in Figure 4, primers from the EGFP gene (Figure 4A)

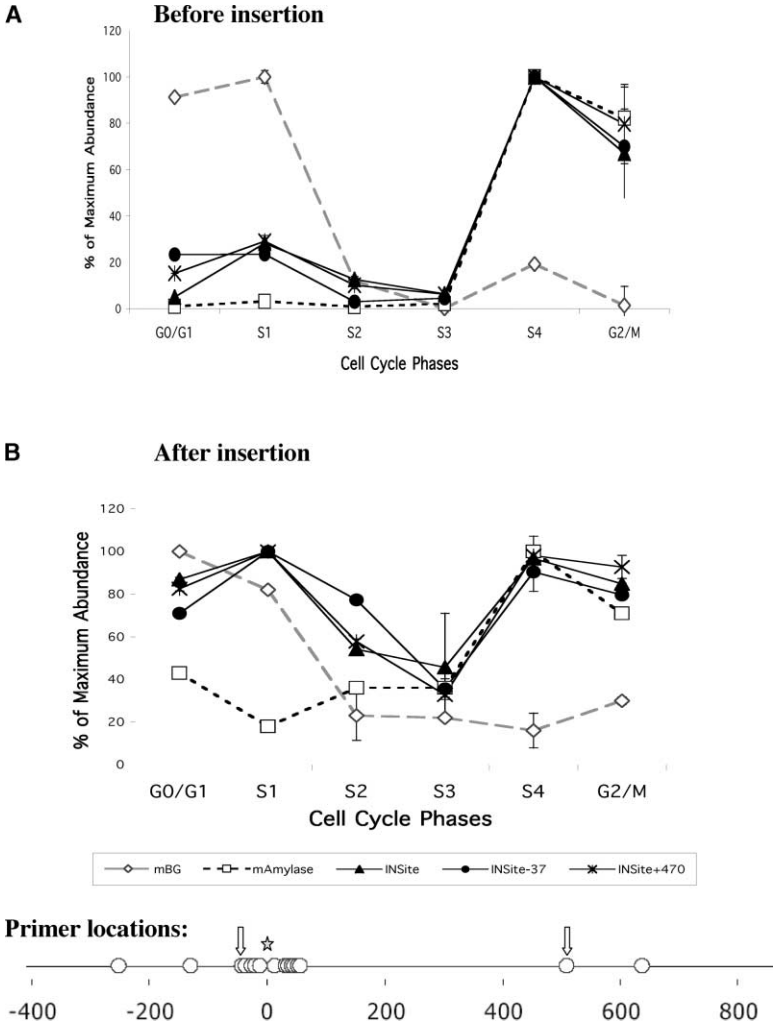

Figure 2. Replication Timing of the Insertion Site before and after Transgene Transfection

The abundance of genomic sequences in BrdU-substituted DNA from different cell cycle fractions was determined by real-time PCR as illustrated in Figure 1B.

(A) Replication timing in MEL cells prior to insertion (Figure 1A, I). Sequences from the murine β-globin locus were more abundant in the G1 and early S phase fractions, whereas the amylase and insertion site sequences were more abundant during the later stages of S phase and in the G2 fraction. The combinations of primers/probes used were: mBG, murine β-like major β-globin; mAmylase, murine amylase gene; and three sequences flanking the insertion site, INsite, INsite−37, and INsite+470. Sequences of all these primers are listed in Table S1. The locations of the the primers adjacent to the insertion site are illustrated in the bottom panel: a star indicates the position of the Insite, and two arrows indicate the positions of the other two primers (INsite−37 and INsite+470, from left to right). Data are expressed as the percentage of the maximum abundance for each probe, ±SEM. The graphs show data from a single representative experiment. Each experiment was performed at least three times for each primer/probe combination at each cell cycle fraction. The data used to create this plot are shown in Table S2. An example of results and data processing is shown in Figure S1.

(B) Replication timing in MEL cells harboring the hyg-TK insertion (Figure 1A, II). The replication timing of sequences from the murine β-globin and amylase loci is similar to that observed in (A). Sequences adjacent to and flanking the insertion site (same as in [A]) were abundant in both early- and late-replicating fractions, suggesting that the two alleles of insertion sites replicate at different times during S phase. These data were obtained from cells growing for several months without drug selection; this finding suggests that the altered replication timing following the insertion of the transgene was a stable phenotype.

preferentially amplified sequences from BrdU-substituted DNA from early S phase; this finding suggests that the transgene replicated early during S phase. In the silent orientation, the EGFP sequence was preferentially amplified in the late S phase fractions. The insertion site (Figure 4B) showed a late replication pattern in the silenced orientation and a mixed early/late pattern in the permissive orientation. The murine β-globin locus exhibited a pattern consistent with early replication (Figure 4C), and the murine amylase locus was preferentially amplified in the later S phase fractions (4D) in cells harboring the transgenes in both orientations.

Changes in Histone Acetylation Correlate with Altered Replication Timing

To investigate whether reversing the orientation of the transgene could alter chromatin structure at a local level, we used chromatin immunoprecipitation assays with antibodies against acetylated histones. We measured the enrichment of specific sequences in the acetylated chromatin fraction by real-time PCR analysis ([29]; for an example, see Figure S3). As shown in Figure 5, transgene-specific sequences (EGFP and human β-globin promoter) were enriched in DNA preparations from chromatin that contained acetylated histones H3 and H4 when the transgene was inserted in the permissive orientation (Figures 5A and 5B). In contrast, transgene-specific sequences were not enriched in acetylated chromatin when the transgene was inserted in the silenced orientation. Sequences from the transgene that harbored a deletion in the β-globin promoter had also exhibited acetylation when inserted in the permissive orientation; this finding suggests that acetylation does not require transcription from the globin promoter. As expected, acetylation of histones H3 and H4 was not detected at the amylase locus but was detected in sequences from the murine β-globin locus, in agreement with previous studies [30]. Sequences flanking the inserted transgenes were not enriched in chromatin preparations containing acetylated histones (5C and 5D).

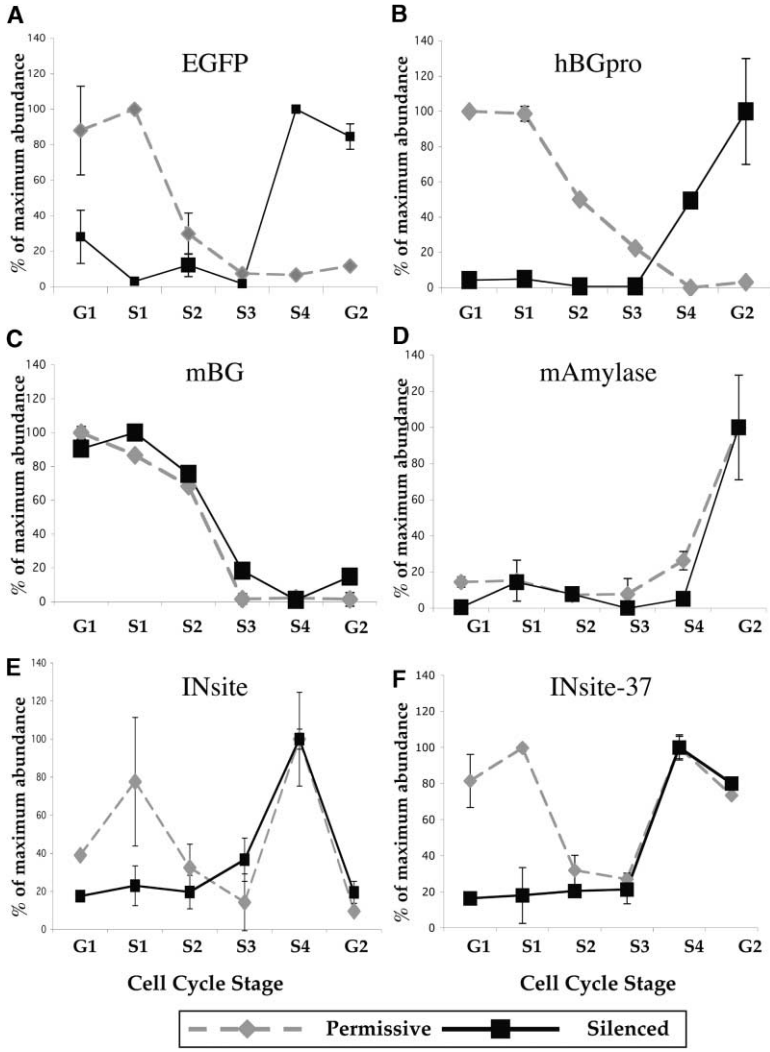

Figure 3. Replication Timing of a β-Globin LCR-Promoter-EGFP Transgene after Recombinase-Mediated Cassette Exchange

The abundance of transgene and genomic sequences in cell cycle-fractionated samples was measured as described in the legend to Figure 1B. In all panels, gray diamonds represent data points from cells harboring transgenes in the permissive orientation (Figure 1A, III), whereas black squares represent data points from cells harboring transgenes in the silent orientation (Figure 1A, IV).

(A and B) Sequences from the transgene (EGFP probes in [A] and hGloPro probes in [B]).

(C) Sequences from the murine β-globin major promoter (mBGmaj).

(D) Sequences from the murine amylase.

(E and F) Sequences from two murine probes adjacent to the insertion site (INsite and INsite−37). Similar data were obtained with other primer/probe combinations (Figure S2 and data not shown).

Methylation of histone H3 on lysine 4 followed the pattern of histone H3 acetylation (data not shown). These data suggest that early replication of the transgene is correlated with modifications of histone tails.

Orientation-Dependent Changes in Replication Timing Do Not Depend on Initiation of DNA Replication within the Transgene

Since the globin promoter included in the transgene originated from the replication initiation region (IR) within the human β-globin locus, we determined whether this region was able to initiate replication at the insertion site. This strategy employed λ-exonuclease to digest all DNA, except strands primed by RNA, as illustrated in Figure 6A [31, 32]. Combined with size fractionation and real-time quantitative PCR, this method allowed us to determine whether sequences from the transgene were present in origin-proximal, newly replicated DNA strands [33]. As shown in Figure 6B, only primers from origin-proximal sequences in the murine adenosine deaminase (ADA) locus or from the murine β-globin IR were able to amplify PCR products, while sequences from the murine globin LCR, which does not contain a replication origin, were not amplified. These data indicated that the nascent strand preparation contained primarily origin-proximal sequences. No products were detected by using primer pairs derived from within the transgene. These data suggested that the DNA fragment derived from the globin IR (Figure 6C) was not sufficient to confer initiation of DNA replication within the transgene. These findings demonstrate that replication did not initiate within the transgene and suggest that initiation of DNA replication from within the transgene was not required for the orientation-dependent replication timing phenotype.

Discussion

We analyzed the replication timing patterns of transgenes, which exhibit orientation-dependent gene silencing in MEL cells. These transgenes are transcribed when inserted in one (permissive) orientation but are silenced when inserted in the opposite orientation. Our data showed that the insertion site was located within a late-replicating chromosomal domain. Insertion of a constitutively transcribed expression cassette at that site facilitated earlier replication of an extended region containing

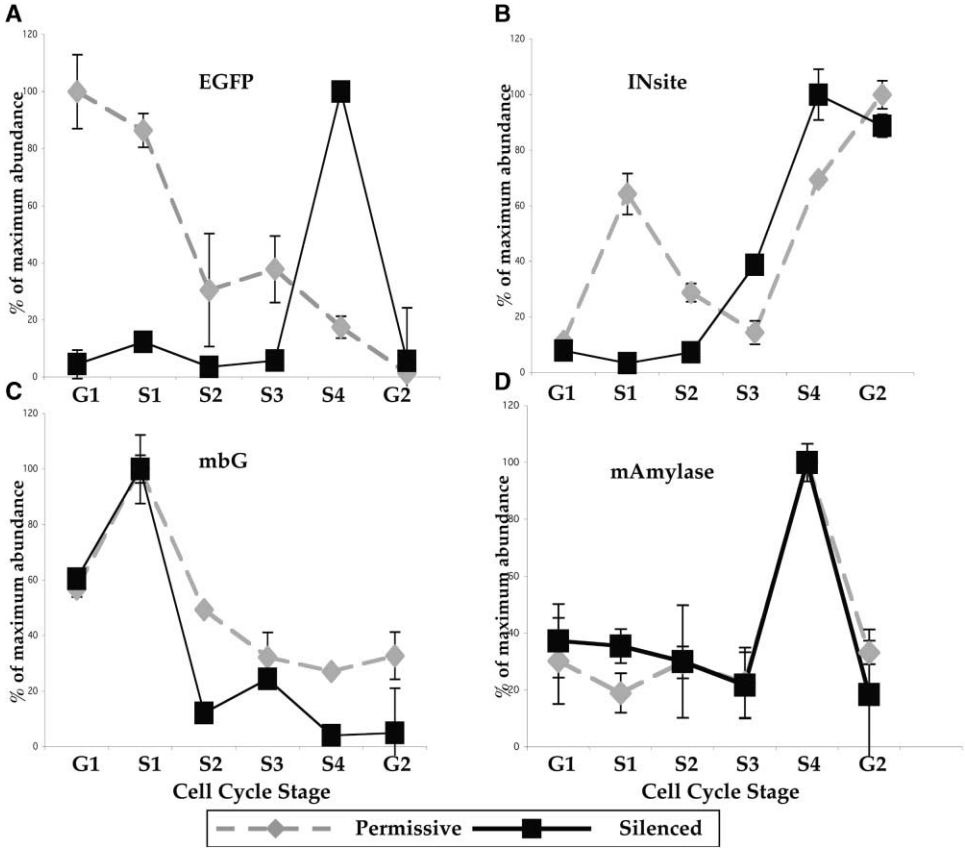

Figure 4. Replication Timing of a β-Globin LCR-EGFP Transgene, which Does Not Contain the β-Globin Promoter

The abundance of transgene and genomic sequences in cell cycle-fractionated samples was measured as described in the legend to Figure 1B. In all panels, gray diamonds represent data points from cells harboring transgenes in the permissive orientation (Figure 1A, V), whereas black squares represent data points from cells harboring transgenes in the silent orientation (Figure 1A, VI).

(A) Sequences from the transgene (EGFP probe).

(B) Sequences from the vicinity of the insertion site (INsite).

(C) Sequences from the murine β-globin major promoter.

(D) Sequences from the murine amylase. Similar data were obtained with other primer/probe combinations (data not shown).

the transgene. The other chromosomal allele, which did not harbor a transgene, remained late replicating. The inserted transgene and the surrounding sequences continued to replicate early when the expression cassette was exchanged with sequences from the human β-globin locus in the permissive orientation. By contrast, early replication was reversed when the transgene containing the human β-globin sequences was inserted in the silencing-prone orientation. Early replication was accompanied by histone acetylation of the transgene region, whereas silenced, late-replicating transgenes did not exhibit such acetylation. Histone acetylation was limited to the transgene region and did not spread to sequences flanking the insertion site, similar to intergenic regions in other early-replicating loci in mammalian cells. Early replication and histone modifications of the transgene did not require the β-globin promoter and did not require initiation of DNA replication from the transgene.

Previous studies have suggested that replication timing zones are extensive chromosomal regions and that replication timing changes occur gradually between early- and late-replicating zones [2, 34, 35]. Our studies demonstrate that a late-replicating chromosomal region can replicate earlier following transgene insertion, in correlation with changes in chromatin structure that facilitate gene expression. These data may seem to contrast with the view of replication timing zones as gradually transitioned, stable chromosomal features. However, previous studies had provided some evidence suggesting that the determination of replication timing is a dynamic process that is responsive to tissue-specific signals and sensitive to chromosomal locations. For example, the human β-globin [3, 5] CD8 [17] and the murine immunoglobulin heavy chain [36] loci exhibit tissue-specific replication timing. In addition, although early replication was formerly thought to be a hallmark of transcribed regions, some transcribed genes were recently shown to reside in transition zones between early- and late-replicating regions [35]. Sequences within these transition zones may be particularly susceptible to tissue-specific changes in replication timing (M. Lalande, personal communication). Our studies show that replication timing can respond to genetic and epigenetic changes in chromatin without cellular differentiation and support the notion that replication timing zones are more dynamic than previously suggested.

At the murine site analyzed in this study, transgene

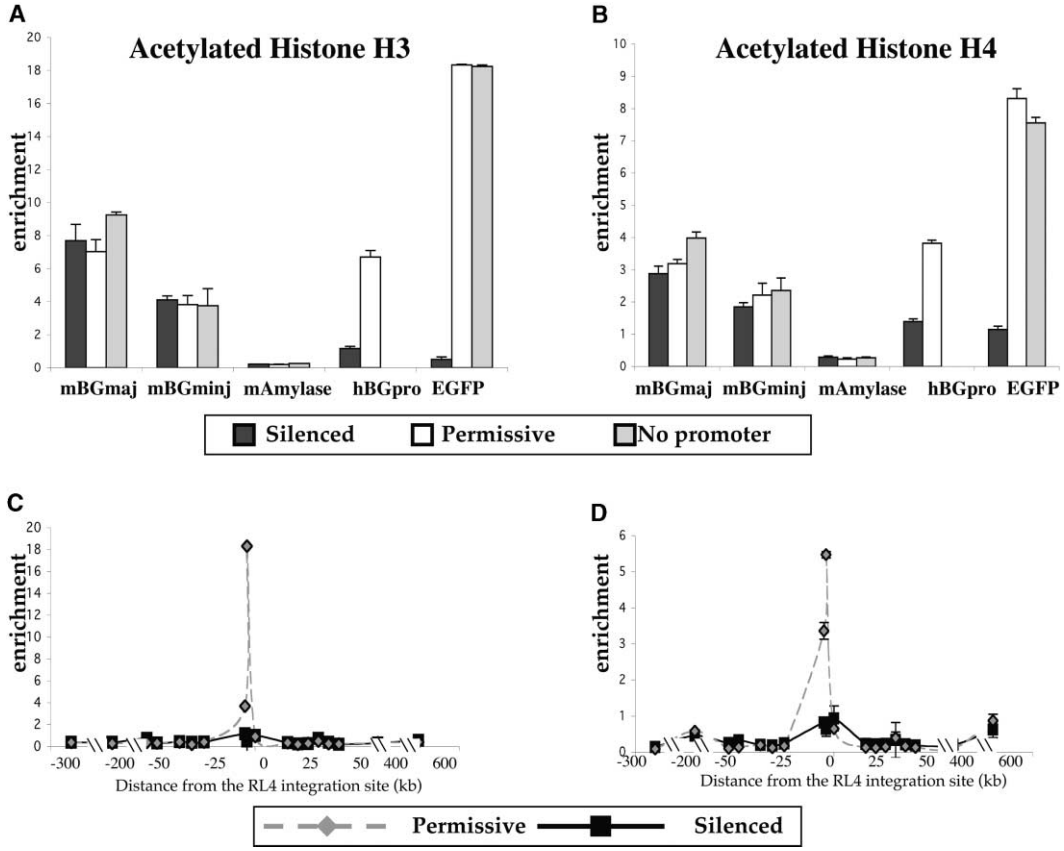

Figure 5. Chromatin Structure at the Insertion Site

(A) The abundance of the transgene and other genes in chromatin that had been immunoprecipitated by an anti-acetylated histone H3 antibody. An example of data processing is shown in Figure S3.

(B) The abundance of the transgene and various other genes in chromatin that had been immunoprecipitated by an anti-acetylated histone H4 antibody. Dark bars, silenced orientation (Figure 1A, IV); light bars, permissive orientation (Figure 1A, III); gray bars, a transgene containing no promoter inserted in the permissive orientation (Figure 1A, V). The probes used included: hBGpro, human β-globin promoter (in the transgene); EGFP, the transgene EGFP gene; INsite, 2 kb 5′ to the insertion site; mAmylase, murine amylase locus; CycD1, the murine locus for cyclin D1; mBmaj and mBmin, sequences from the β globin major and minor regions, respectively.

(C) The pattern of histone H3 acetylation in the region straddling the insertion site on murine chromosome 15.

(D) The pattern of histone H4 acetylation in the region straddling the insertion site on murine chromosome 15. Diamonds indicate data points from chromatin prepared from cells containing the transgene in the permissive orientation, while squares indicate data points from chromatin prepared from cells containing the transgene in the silenced orientation. For primer/probe sequences and map positions, see Table S1 and the illustration at the bottom of Figure 2.

sequences that included a strong promoter were sufficient to shift the replication timing of chromatin from later to earlier periods during S phase. However, several lines of evidence suggest that promoter strength is not the sole determinant of the extent of gene expression and replication timing. First, maintenance of the early-replicating domain was orientation dependent, similar to position-dependent transgene silencing events frequently observed in gene therapy studies [37]. These position effects suggest that the inserted sequences cannot act as the sole determinants of replication timing and must interact with the flanking sequences to modify chromatin structure. Second, we have observed that the globin promoter was not necessary for early replication and histone acetylation. Although our studies did not formally exclude possible low-level transcription originating from nonpromoter sequences in the transgene or flanking sequences, these data suggested that recruitment of the transcription machinery to the inserted globin promoter did not play a role in establishment of the early-replicating domain. Finally, insertion of very strong promoters in other loci may fail to overcome transcriptional silencing, as was recently reported for the inactive X chromosome [38]; again, this finding suggests a role for flanking sequences in the establishment of a replication timing domain in association with transgene insertion. These observations are consistent with data obtained from LCR mutants at the native human β-globin locus that show that early replication correlates with a decondensed chromatin structure, not with the level of transcription per se [25, 26, 39]. Our data, together with the above-mentioned studies, imply that replication timing domains are established dynamically in response to variations in chromatin condensation, which can be triggered either by differentiation or by changes at the DNA sequence level.

The notion that replication timing domains can be established in a dynamic and reversible manner is con-

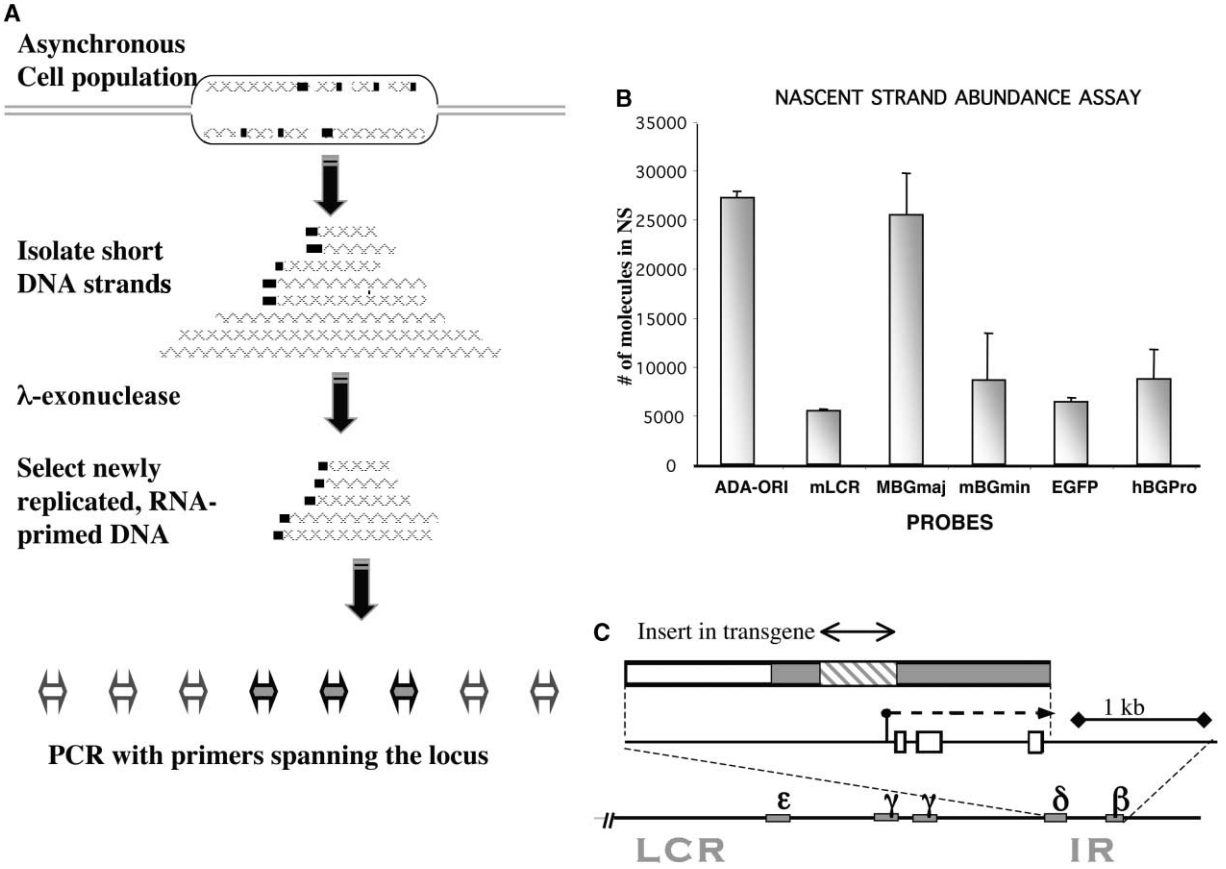

Figure 6. Nascent Strand Abundance Analysis

(A) A schematic illustration of the method used to analyze the abundance of nascent DNA strands [32]. Newly replicated DNA is isolated in two steps. First, short DNA strands are separated from the rest of the genome by sucrose gradient fractionation. λ-exonuclease is then used to digest all short DNA fragments that are not primed by RNA. The undigested material consists of newly replicated, RNA-primed DNA strands. Real-time PCR with primers encompassing specific regions of interest allows primers from regions adjacent to replication initiation sites to amplify abundant product (filled arrows), whereas primers that lie far from initiation regions will amplify poorly (empty arrows).

(B) Results of a nascent strand abundance assay with genomic and transgene sequences. Primers from the murine ADA replication origins and from the murine β major region in the β-globin locus, which lies within an initiation zone, amplified abundant product in the nascent strand preparation. Primers from regions that are distal to replication origins, such as the murine LCR, exhibited low amplification from the same nascent strand preparation. Primers from the transgene showed that these sequences were present in low abundance, which is consistent with an absence of initiation events within the transgene sequences. These data were obtained from cells harboring the transgene in the permissive orientation (Figure 1A, III), but similar results were obtained from cells harboring the transgene in the opposite orientation.

(C) The location of the inserted globin promoter sequences relative to the published human β-globin replicator. The bottom line is a map of the human β-globin locus that indicates the position of the initiation region (IR) and the locus control region (LCR). The β-like globin genes are indicated as boxes. The middle line is an enlarged schematic of the IR showing the positions of the globin promoter (arrow) and exons (boxes). The upper box is drawn to the same scale as the middle line and represents the minimal replicator region identified by ectopic replication analyses within the IR. The gray box within the replicator delineates the boundaries of a region that was identified as essential, but not sufficient, for replicator activity. The hatched region represents the extent of the IR sequences inserted into transgenes (Figure 1A, III and IV) used in this study.

sistent with the observation that the timing of DNA replication is determined anew every cell cycle in both mammalian cells [40] and yeast [41]. At the molecular level, such changes may be mediated through components of the prereplication complex, which also bind components of heterochromatin [42, 43] and play a role in transcriptional silencing in yeast [44]. The coregulation of replication timing and chromatin structure, observed here, is consistent with data suggesting that initiation requires not only the origin recognition complex but also a specific nucleosome structure near the replication origin [45] and that mutants in DNA polymerase exhibit an altered positioning of chromatin components such as Swi6 [46]. An intriguing suggestion is that late replication of condensed chromatin contributes to the preservation of the condensed state through a positive feedback loop that depends on the lack of histone acetylation during late S phase [1]. Further characterization of the dynamics of gene activation and silencing in our system are likely to shed light on the causative relationship between replication timing and gene expression.

The transgene constructs used in this study did not include an active replication origin, yet the insertion of the transgene was able to alter replication timing. These findings imply that the sequence determinants of repli-

cation timing may be distinct from the determinants of replication initiation sites. In budding and fission yeast, replication origins and the sequence determinants of replication timing reside in close proximity to each other but can still be separated by careful molecular dissection [8, 47]. A similar situation may exist in the human β-globin locus, where the LCR may act as a chromatin modifier required to set the stage for early replication under the appropriate differentiation conditions [21, 48] but is not required for early replication from the globin origin at ectopic locations [23]. Our studies, as well as the studies in the native globin locus, imply that the sequence determinants of replication timing act through epigenetic chromatin modifiers and do not require direct interaction with replication origins.

Supplemental Data

Supplemental Data including a description of the Experimental Procedures used in this study, a list of primers and probes (Table S1), examples of derivation of replication timing and acetylation measurements from raw data (Table S2 and Figures S1 and S3), and an analysis of replication timing for additional probes in the vicinity of the RL4 insertion site (Figure S2) are available at http://www.current-biology.com/content/supplemental.

Acknowledgments

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